Strategy for nuclear-magnetic-resonance-based metabolomics of human feces

Lamichhane, Santosh; Yde, Christian Clement; Schmedes, Mette Søndergaard; Max Jensen, Henrik; Meier, Sebastian; Bertram, Hanne Christine

Published in:
Analytical Chemistry

Link to article, DOI:
10.1021/acs.analchem.5b00977

Publication date:
2015

Document Version
Peer reviewed version

Strategy for nuclear-magnetic-resonance-based metabolomics of human feces

Santosh Lamichhane[1], Christian C. Yde[1], Mette Søndergård Schmedes[1], Henrik Max Jensen[2], Sebastian Meier[3], and Hanne Christine Bertram[1]*

[1]Department of Food Science, Aarhus University, Kirstinebjergvej 10, DK-5792 Aarslev, Denmark.

[2]DuPont Nutrition Biosciences ApS, Edwin Rahrsvej 38, 8220 Brabrand, Aarhus, Denmark

[3] Technical University of Denmark, Department of Chemistry, Kemitorvet, Building 201, DK-2800 Kgs. Lyngby, Denmark

* Corresponding author. Email: hannec.bertram@food.au.dk

Abstract:

Metabolomic analyses of fecal material are gaining increasing attention because the gut microbial ecology and activity have an impact on the human phenotype and regulate host metabolism. Sample preparation is a crucial step, and in this study we recommend a methodology for extraction and analysis of fresh feces by NMR-based metabolomics. The evaluation of extraction solvents showed that buffer extraction is a suitable approach to extract metabolic information in feces. So, the effects of weight-to-buffer (Wf:Vb) combinations and the effect of sonication and freeze-thaw cycles on the reproducibility, chemical shift variability, and signal to noise ratio (SNR) of the ^{1}H NMR spectra were evaluated. Based on our results, we suggest that fresh fecal extraction with a Wf:Vb ratio of 1:2 may be the optimum choice to determine the overall metabolite composition of feces. In fact, more than 60 metabolites have been assigned in the NMR spectra obtained from the fresh fecal buffer extract, and assignments of the lipophilic signals are also presented. To our knowledge, some of the metabolites are reported here for the very first time employing ^{1}H NMR spectroscopy on human fecal extracts.

Keywords: Nuclear magnetic resonance, metabolomics, gut, human feces, metabolites

Introduction

Metabolomics is a holistic approach to study systematic metabolic changes in biological samples. In general, nuclear magnetic resonance (NMR) and mass spectrometry (MS) are the analytical techniques used to observe and quantify metabolites from biological samples, and each technique has its own advantages and disadvantages. NMR-based metabolomics has emerged as a reliable high throughput analytical technique that has been extensively applied to a variety of biological samples such as urine, plasma, tissues, saliva, milk, seminal fluid, and sputum.[1-4] Presently, urine and plasma are the most common sample types included in metabolomics studies. However, metabolomics on fecal material is gaining increased attention because gut ecology has an impact on the human phenotype and gut-microbe cross talk is regarded as one of the key determinants in the regulation of host metabolism.[5-11] Because feces comes into direct contact with the colon and sample collection is non-invasive, feces is the most biologically most relevant sample material for metabolomics studies aiming to explore gut microbial activity and thereby gut health status. To date, NMR studies focused on fecal metabolomics have been applied to gain insight into metabolic effects involved in dietary interventions [12,13] and to study gut diseases such as inflammatory bowel disease, [14,15] ulcerative colitis, [14] and colorectal cancer.[16] An increased understanding of the metabolic activity in the colon may contribute to the identification of new biomarkers that could be related to health or disease status and molecular regulation of the complex gut system.

In order to extract as much information as possible from a complex fecal sample, optimization and standardization of an analytical methodology are essential. Although several studies have been reported on fecal metabolomics, [12,14-16] few studies have focused on optimizing the NMR methodology for fecal extracts and detailed spectral assignments have rarely been reported. [17-19] Two of the most important factors to consider in the optimization of an extraction method are (i) the

production of reproducible NMR spectra with low inter-sample chemical shift variability and (ii) high signal-to-noise ratio (SNR). In addition, the extraction method should preferably be rapid, robust, (and suitable) and compatible with high-throughput analyses. Previously, fecal sample extractions have been performed by centrifugation, [20] D_2O, [21] distilled water, [16] buffers, [14,22] and mixed solvent extractions on lyophilized samples.[18] Lyophilization is advantageous, as it minimizes the effect of solvent dilution (water content) on the feces. However, lyophilization affects the concentration of volatile metabolites, such as short chain fatty acids and succinate.[18,19] Therefore, to reduce the loss of volatile metabolites and minimize the steps required during extraction; fresh fecal samples are considered the preferred choice for fecal metabolomics studies. Buffer extraction might be the most suitable approach to extract metabolic information as it reduces inter-sample pH variation during extraction. In order to obtain reproducible NMR spectra with lower inter-sample chemical shift variability and high SNR, the choice of extraction solvents must be evaluate and the fecal weight-to-buffer volume (Wf:Vb) must therefore be optimized.

In previous studies, different extraction solvents were implied for extraction[16,18-20] and Wf:Vb ratios of 1:50,[14] 1:5[16], 1:10,[23] 1:2 and an unknown ratio [15,17,22] have been applied, but none of the studies report the rationale for their choice of solvent and the weight-to-buffer volume. Wu et al. [24] have recommended the use of a Wf:Vb ratio of 1:10 and the pooling of the first two extracts. It is also noteworthy that the recommendation (Wf:Vb of 1:10) was solely based on evaluation of chemical shift inconsistency and the experiment was conducted in fecal material from mice. Saric et al. showed that the fecal metabolome varies between species and, more specifically, the human metabolome contained lower levels of lactate and amino acids compared to mice and rats.[19] Thereby, this finding urges further investigation of the choice of fecal weight-to-buffer ratio to extract metabolites from fresh human feces in metabolomic analyses. Besides that, the fecal

metabolomics studies have focused on the analysis of intracellular bacterial metabolites; therefore, different sample pretreatment techniques have been used to homogenize fecal samples. However, the presence of different residual food components in the feces might affect the extraction. Therefore, it is of interest to compare the effects of sample pre-treatment and the choice of weight-to-buffer ratio during metabolic profiling from fresh human samples. In the present study we aimed to compare the effect of different solvents during extraction, weight-to-buffer combinations on the reproducibility, chemical shift variability, overall metabolite composition and SNR of the NMR spectra from healthy human feces. In addition, we analyzed the consistency of fecal metabolite composition upon different sample treatment techniques (sonication and freeze-thaw cycles) and suggest an optimized protocol for NMR-based metabolomics analyses of human feces.

Materials and methods

Sampling and sample material

Fecal material from nine healthy volunteers was used for the study. The study protocol was approved by the Ethics and Research Committee and conducted according to the guidelines in the Declaration of Helsinki. The fecal material was collected from all subjects in a falcon tube immediately after defecation. The samples were then stored at -20 °C prior to analysis. Initially, fecal samples from four healthy volunteers were used to optimize the fecal-to-water buffer ratio during extraction. Samples from an additional five subjects were included to elucidate the reproducibility of the method and the stability over time.

Fecal metabolite extraction

Fecal water was extracted to ratios of 1:2, 1:5, and 1:10 (weight of fresh feces-to-buffer) in 0.75 M phosphate buffered saline (PBS, pH 7.4). The samples were homogenized by whirl mixing for 2 min, and then aliquots were centrifuged at 10,000 *g* for 15 minutes at 4 °C (Eppendorf 5471, USA). The supernatants were carefully removed and stored in Eppendorf tubes at -80 °C until analysis. To determine recovery, feces from one individual (n = 3) was spiked with 100 μL of standards maleic acid, dimethyl sulfone, and succinic acid (0.05 M). Fecal water was extracted to ratios of 1:2, 1:5, and 1:10 (w/v) as described earlier. The recovery values of dimethyl sulfone, and succinic acid were referenced to maleic acid. In addition, to investigate the influence of solvent during fecal metabolites extraction, feces from one individual has been extracted in duplicate with distilled water, D_2O extraction, (adapted from Monleón et al.[16]) and using combination of methanol/chloroform/water (section A, supplementary information).

99 **Freeze thaw and sonication treatment**

100 To compare the effect of sample pretreatment on the fecal NMR profile; we extracted feces
101 simultaneously with/without freeze-thaw and sonication treatment. During freeze thaw whirl-mixed
102 samples were frozen to −18 °C for 10 minutes and immediately thawed at 4 °C for 10 min in
103 Eppendorf tubes (Eppendorf 5471, USA). This treatment was replicated for 3 cycles. Sonication
104 was conducted between each freeze thaw cycle (Branson 5210, USA). The samples were placed in
105 Eppendorf tubes and sonicated for three consecutive cycles of 2 min on and off between each freeze
106 thaw cycle.

107 **pH measurement**

108 The pH values of the fecal extracts diluted to 1:2, 1:5, and 1:10 (w/v) in 0.75 M phosphate buffered
109 saline (PBS, pH 7.4) were measured at room temperature (22 °C) before NMR analysis, using pH
110 electrodes for NMR sample tubes (IKATRON®, Germany).

111 **^{1}H NMR spectroscopic analyses**

112 Fecal water samples extracted in PBS were thawed and centrifuged at 10,000 *g* for 15 minutes at
113 4°C (Eppendorf 5471, USA). A volume of 500 μL of clear supernatant was transferred to a 5 mm
114 NMR tube, and 100 μL of deuterium oxide (D_2O) containing 0.025 mg/mL of 3-(Trimethylsilyl)
115 propionic acid-*d*4 sodium salt (TSP) was added as a lock solvent. One-dimensional NMR
116 experiments were carried out using a Bruker Avance III 600 MHz spectrometer (Bruker,
117 Rheinstetten, Germany) equipped with a 5 mm triple resonance (TXI) probe at 298 K. A standard
118 Bruker noesypr1d (90°-t_1-90°-d_{mix}-90°-FID) sequence was used to suppress signals from water
119 molecules, where t_1 is a 4 μs delay time and d_{mix} is the mixing time. Acquisition parameters for the
120 spectra were 64 scans, a spectral width of 7288 Hz collected into 32K data points, an acquisition
121 time of 2.24 s and an inter-scan relaxation delay of 5 s. The Free Induction Decay (FID) obtained

was multiplied by 0.3 Hz of exponential line broadening before Fourier transformation. The spectra were referenced to TSP (chemical shift 0 ppm), phased, and baseline corrected in Topspin 3.0 software (Bruker, Rheinstetten, Germany). SNR was measured using the built-in graphical interface in Topspin 3.0, where alanine (1.47 ppm) was considered to be the signal, and spectra at lower frequency than 0 ppm were considered to represent the noise region. Relative quantification of selected ^{1}H resonance was performed by integration of peak areas using Topspin 3.0 (Bruker). Assignments of ^{1}H NMR signals were carried out using Chenomx NMR Suite 7.7 (Chenomx, Canada) according to the Human Metabolome Database,[25] and literature.[14,18,26] In addition, heteronuclear single quantum coherence (HSQC) and total correlation spectroscopy (TOCSY) 2D NMR techniques were applied to confirm the identity of the assigned metabolites (see Figures S1 to S6 in supplementary material). The HSQC experiment was acquired with a spectral width of 7288 Hz in the ^{1}H dimension and 27164 Hz in the ^{13}C dimension, a matrix with size of 4096×1024, 512 transients per increment and a relaxation delay of 2 s. The TOCSY spectrum was acquired with acquired with a spectral width of 7288 Hz in the both ^{1}H dimension, a matrix with size of 2048×512, and an inter-scan relaxation delay of 2s. In addition, two-dimensional NMR experiments were carried out using a Bruker Avance III 800 MHz spectrometer equipped with a 5-mm 1H observe cryoprobe (Bruker Biospin, Rheinstetten, Germany). The HSQC experiment was acquired with a spectral width of 11961 Hz in the ^{1}H dimension and 33276 Hz in the ^{13}C dimension, a matrix with a size of 4096×4096, 512 transients per increment and a relaxation delay of 1.5 s. The TOCSY spectrum was acquired with a spectral width of 9578 Hz in both ^{1}H dimensions, a matrix with a size of 8192×1536, and an inter-scan relaxation delay of 1.8 s.

144 **Multivariate Data analysis**

145 ^{1}H NMR spectra were imported to Matlab R2010b (The Mathworks, Inc., USA), and the
146 misalignments of the spectra were corrected using the icoshift algorithm, based on the correlational
147 shifting of spectral intervals.[27] The spectrum with the highest correlation to the rest of the spectra in
148 the matrix was used as a reference. The 9.5 to 12 ppm and 0.5 to -1.2 ppm regions and the region
149 containing residual water resonance (4.8. to 4.7 ppm) were removed from the aligned spectra. The
150 spectra were normalized to unit area and pareto-scaled before principal component analysis (PCA)
151 using the PLS Toolbox (Eigenvector Research, USA) in MATLAB20010b.

152

Results and discussion

The ^{1}H NMR spectra obtained from fresh fecal samples revealed the presence of a wide range of metabolites. Figure 1 shows a representative spectrum of a fecal sample extracted in PBS buffer. The obtained ^{1}H NMR spectra contained resonances from short chain fatty acids (predominantly acetate, propionate, and butyrate), branched-chain fatty acids (iso-valerate, iso-butyrate), biogenic amines (trimethylamine and dimethylamine), organic acids (succinate, fumarate), carbohydrates (glucose predominant), and amino acids (leucine, isoleucine, valine, tyrosine, phenylalanine, and others). Figure 1 and the numbering scheme outlined in Table 1 summarize the assigned resonances of the fresh fecal samples. To the best of our knowledge, we report here for the first time signals from the metabolites xylose, arabinose, nicotinate, orotic acid, malic acid, xanthine, thymine, and acetoin in the fecal extract using ^{1}H NMR spectroscopy.

Evaluation of extraction solvents

The influence of solvent: water, D_2O, PBS buffer, and mixed solvent (methanol/chloroform/water) on extraction of the fecal metabolites was evaluated by assessing the metabolite compositions in the NMR profile obtain from the different extracts. This analysis revealed that the signals from n-acetyl compounds, lipid residue, and some unknown anomeric hydrogen in carbon signals 5.0-5.5 ppm were preferentially extracted in the methanol extract (Figure S7, supplementary information). In contrast, the resonance from trimethylamine appeared only in the ^{1}H NMR spectra of the extracts from distilled water, D_2O, or PBS buffer. Signals from SCFA, amino acids, and organic acids were similar in the four extracts, however, recoveries differed.

The methanol/chloroform/water extraction is time-consuming, labor-intensive, expensive, and toxic (hazardous) compared to the one-solvent extractions. As PBS buffer extraction reduces inter-sample pH variation during extraction, we suggest PBS buffer as the best choice for extraction

176 solvent. However, the lipid-containing organic (chloroform) phase in the
177 methanol/chloroform/water provides complementary information about the non-polar metabolites in
178 the feces, which presently has not been investigated thoroughly by using NMR-based
179 metabolomics. The NMR signals recorded on chloroform phase in the non-polar extracts were
180 assigned to different lipid classes/functional groups. Our result indicates the presence of unsaturated
181 fatty acids, triacylglycerides, glycerol backbone of phospholipid, phosphatidylcholine, alkyl chains,
182 and acyl saturated chains in the proton NMR spectra of the lipid extracts. These signals
183 predominantly from the saturated and unsaturated fatty acids are comparable to the lipid signals in
184 the other body fluid such as plasma and the tissue samples. Assignments of ^{1}H NMR lipid signals
185 were carried out using the American Oil Chemist Society (AOCS) Lipid library [28] and literature.
186 [29,30] In future, examination of these fatty acid compositions in feces might have great potential to
187 reveal mechanistic interplay between the gut microbiota and lipid metabolism.

188

189 **Effect of varying the Wf:Vb ratio during extraction**

190 Theoretically, concentrated samples are beneficial in NMR spectroscopy as SNR will increase with
191 increasing amount of compound. However, in metabolomics concentrated samples at the same time
192 retain a challenge as they potentially may lack signal stability due to pH variations and
193 intermolecular interactions. Therefore, in the present study the possible tradeoffs of chemical shift
194 stability and signal intensity were investigated in a detailed comparison of three buffer ratios
195 (dilutions) and two sample treatments. Three different buffer dilutions (1:2, 1:5, and 1:10) and the
196 effect of sample treatments (sonication and freeze-thaw cycles) during fecal metabolite extraction
197 were evaluated. The effects of sonication and freeze-thaw cycles were less pronounced when
198 compared to the effect of varying buffer concentration on the fecal metabolite extraction (Figure 3).
199 The signal to noise ratio (SNR) measured from the fecal extracts with different Wf:Vb ratios clearly

200 shows that 1:2 extracts have higher SNR in comparison to 1:5 and 1:10 extracts (Figure 3), which
201 can be ascribed to the dilution of metabolites during extraction in the 1:5 and 1:10 samples. Visual
202 inspection of the spectra (scaled by the dilution factor) showed that the intensities of the metabolites
203 and the noise in the spectra were different in the 1:2 extract when compared to the 1:5 and 1:10
204 extracts (Figure 4). The signals from nicotinate, UDP glucose, tryptophan, bile acids are masked by
205 higher contribution from noise in the 1:5 and 1:10 extracts compared to the 1:2 extract (Figure 4). In
206 contrast, the signal from the n-acetyl compound had lower signal intensity in the 1:2 extract
207 compared to 1:5 and 1:10 (data not shown). The lower intensity of n-acetyl compound in 1:2 extract
208 can likely be ascribed to saturation of metabolites during extraction. In addition to signal intensity
209 of metabolites in the NMR spectrum, the recovery of a metabolite during extraction is essential.
210 Consequently, in order to measure recovery, feces were spiked with maleic acid, dimethyl sulfone,
211 and succinic acid. The results showed that the recovery yield of these standards is minor influenced
212 by dilution factor (Table 2). Nevertheless, the overall results implicate that the Wf: Vb affects the
213 detection and quantification of certain metabolites when extracted with a high buffer volume
214 (Figure 4). Thereby, based on our finding it is likely that proper choice of solvent and the weight-to-
215 buffer volume during feces extraction might have an impact in the outcome/understanding of the
216 nutritional metabolomics study.
217

218 **Inter-sample pH consistency**

219 In general, the pH of human feces can range from 6.8 to 7.0, and the variation usually depends on
220 diet, xenobiotic intake and/or the health status of the individual. For effective subsequent data
221 mining, inter-sample pH consistency remains a critical problem because this variability can result in
222 chemical shift variations of the metabolites. Although some peak alignment techniques can
223 minimize the chemical shift inconsistency in the data after NMR acquisition, chemical shift

224 variations still create ambiguity for overlapping and unknown signals.[31] Therefore, the most
225 efficient strategy is to minimize such inter-sample pH variations during sample preparation i.e.,
226 prior to data acquisition. In the present study, we measured the inter sample pH variation for the
227 three different buffer concentrations (1:2, 1:5, and 1:10) and sample treatments (sonication and
228 freeze-thaw). Differences in pH between the samples due to sample treatment were minor in
229 comparison with the effects of the Wf:Vb ratio of buffer (Figure S8, supplementary information).
230 The pH of the fecal sample increased with an increase in the Wf:Vb ratio of buffer. The samples
231 extracted with a Wf:Vb ratio of 1:10 and 1:5 showed more stable pH in the range of 7.30 to 7.45,
232 while the samples extracted with a 1:2 Wf:Vb ratio varied in pH from 7.20 to 7.40. Even though the
233 pH was marginally more stable between the samples extracted with 1:5 and 1:10 Wf:Vb ratios, the
234 SNR was reduced more than 2-fold and, the variations in the chemical shifts for the samples
235 extracted at 1:2 were sufficiently low to be easily overcome by the application of an alignment
236 method such as ico-shift,[27] FOCUS (based in the Recursive UN referenced Alignment of Spectra
237 algorithm) [32] or binning of the sample during data pre-processing (Figure S9, supplementary
238 information). For the purpose of NMR-based metabolomics of the feces, it is essential to extract all
239 the metabolites from fecal samples with substantially higher signal intensity, i.e., reasonable SNR.
240 Thus, it was concluded that a Wf:Vb ratio of 1:2 is the optimum ratio during extraction under the
241 conditions tested. It can be expected that the optimum Wf:Vb ratio will depend on the physico-
242 chemical properties of the fecal material. It is therefore recommended to use the Bristol stool
243 scaling (BSS) [33] of the feces prior to analysis; so that the physico chemical properties of the stool
244 samples are taken in consideration.
245

Reproducibility

To address the suitability of a methodology to profile metabolites in metabolomics studies, the reproducibility of the method should be evaluated. Pareto-scaled normalized NMR spectra obtained at three different time points from 5 individuals (extracted with a Wf:Vb ratio of 1:2) were analyzed by multivariate data analysis to elucidate the reproducibility of the method. The PCA score plot in Figure 5 shows that the individual samples (represented by the colors) were clustered together depicting the reproducibility of the extraction methodology. In addition, the data from this study also revealed distinct inter-individual variation supporting the fact that the sample preparation method was capable of detecting and identifying metabolic differences among individuals. Intriguingly, the samples from two subjects (depicted as green and cyan) did not cluster together as observed for the other three subjects (Figure 5). During extraction, the presence of undigested food residuals were clearly observed in these samples compared to the rest of other samples. The presence of these food residuals could affect the NMR profile of the fecal extract. Therefore, it may be worthwhile to perform BSS which could for instance indicate that the observed differences within the samples may be due to larger variation in the stool consistency of the individuals.

To eliminate inter-individual differences and assess the reproducibility performance of the metabolites, extraction was done in triplicate using different Wf: Vb ratio (1:2, 1:5, and 1:10) from the feces of same individual. The coefficient of variation (% CV) of eleven different metabolites was determined (Figure 6). The majority of the metabolites show low and similar % CV independent of the buffer dilutions. However, fumarate (≈55 %) and formate (≈18 %) displayed considerably higher % CV when extracted at 1:5 and 1:10 dilution ratio respectively, when compared to metabolites extracted with the 1:2 dilution ratio. This could possibly be ascribed to the difference in the pH between the samples extracted in Wf: Vb ratio of 1:10 and 1:5, and 1:2 as seen

previously. This difference most probably affected the recoveries of fumarate and formate, indicating they were preferentially extracted in lower pH. Considering the recovery would be different for specific class of compound, the main purpose for metabolic profiling/metabolomics study is to obtain reproducible spectra that can be on compared with each other. Intriguingly, based on our results, it is also noteworthy that Wf: Vb ratio of 1:2 showed reproducible result during extraction under the conditions tested. Thus, we suggest that the Wf: Vb ratio affects the reproducibility of certain metabolites when extracted with a diluted buffer.

Fecal NMR profile stability study

The NMR experiments may last from a few minutes to hours of acquisition time. During NMR acquisition, the metabolites might change over time due to either enzymatic or non-enzymatic processes. Thus, it is essential to elucidate spectral changes that might occur over time. Therefore, ^{1}H NMR spectra were acquired over a period of 12 hours by repeating the acquisition at regular intervals of 2 hours (Figure S10 and S11). The obtained spectra were analyzed by overlaying the spectra from all acquired time points. The results showed that the metabolite profile of the fecal extracts remained stable over the 12 h period.

Conclusions

Fecal-based metabolomics is a rapidly emerging discipline. An optimized sample preparation is crucial for metabolomics studies, and here we suggest buffer extraction might be the most suitable approach to extract metabolic information in the feces. The study demonstrates that a Wf:Vb ratio of 1:2 might be optimal quality for ^{1}H NMR spectroscopic data for metabolomics data of human feces, while the effects of sonication and freeze-thaw were less pronounced of the condition tested.

More than 60 metabolites have been assigned from the ^{1}H NMR spectra obtained, and to our knowledge, some of the metabolites are reported here for the very first time using NMR spectra acquired on fecal extracts.

Acknowledgement

The present study is part of the Ph.D. work of Santosh Lamichhane and was financially supported by DuPont, Food Future Innovation (FFI), and Aarhus University. The authors also wish to thank the Danish research council FTP for financial support through the project 'Advances in food and nutrition research through implementation of metabolomics technologies' (#274-09-0107)'. The 800 MHz spectra NMR spectra were obtained at the Danish Instrument Center for NMR spectroscopy of Biological Macromolecules.

Supporting Information Available

Supplementary method section on metabolite extraction methods and Figure S1 to S11. This information is available free of charge via the Internet at http://pubs.acs.org/

Figure Captions

Figure 1. Representative ^{1}H NMR spectrum of a human fecal sample extracted in PBS buffer. A) 0.7 to 4.55 ppm; B) 4.9 to 8.55 ppm C) 8.6 to 9.8 ppm. The aromatic region in the spectrum (8.6 to 9.8 ppm) has been magnified three times as compared to the region B. The inserts show additional signals detected in spectra obtained on other human fecal samples. Keys to the figure are given in Table 2.

Figure 2. Representative ^{1}H NMR spectrum of a human fecal sample extracted in organic (chloroform) phase in the methanol/chloroform/water extract. A) 5.7 to 4.7, double bond section; UFA = unsaturated fatty acids; TAG = triacylglycerides, Glyc-B = glycerol backbone of phospholipid. B) 4.4 to 2.8 regions; PC = phosphatidylcholine. C) Alkyl and Acyl saturated chains

Figure 3. Average SNR ratios from the fecal extracts with different Wf: Vb and obtained with sonication and without sonication, respectively (n=4). The alanine signal at 1.44 ppm was used for the calculation of SNR. Error bars show standard deviations.

Figure 4. Representative ^{1}H NMR spectra of human fecal extracts with varying Wf: Vb ratios A) 1:10 B) 1:5 C) 1:2.

Figure 5. PCA scores plot showing reproducibility for fecal extracts with Wf: Vb ratio 1:2. Each symbol represents an individual. The number 1, 2, 3 illustrates the samples from same individual collected during 3 different time periods.

Figure 6. The coefficient of variation (% CV) of eleven different metabolites from NMR measurement of the fecal extract (three different buffer dilutions 1:2, 1:5, and 1:10).

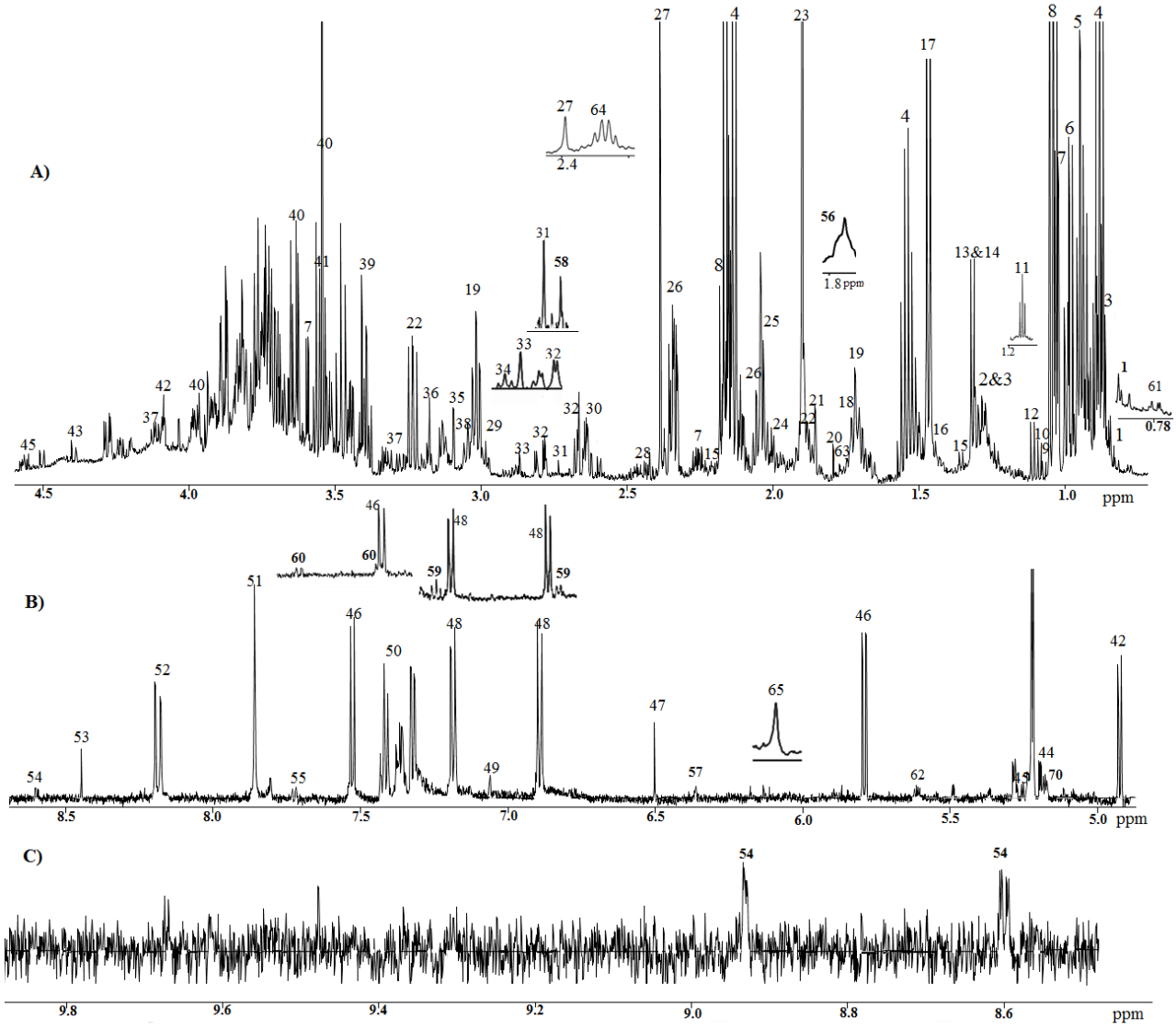

Figure 1.

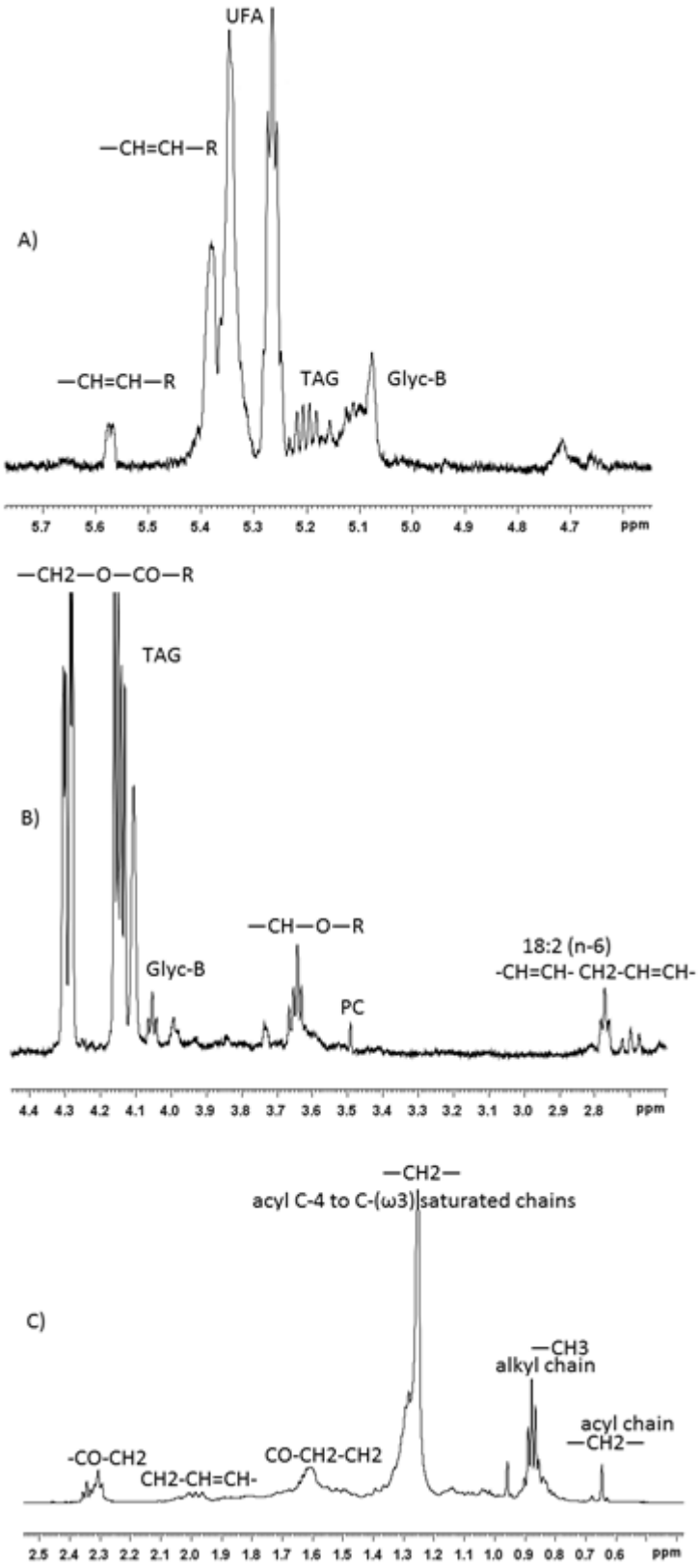
A)
UFA
—CH=CH—R
—CH=CH—R
TAG
Glyc-B
5.7 5.6 5.5 5.4 5.3 5.2 5.1 5.0 4.9 4.8 4.7 ppm
B)
—CH2—O—CO—R
TAG
Glyc-B
—CH—O—R
PC
18:2 (n-6)
-CH=CH- CH2-CH=CH-
4.4 4.3 4.2 4.1 4.0 3.9 3.8 3.7 3.6 3.5 3.4 3.3 3.2 3.1 3.0 2.9 2.8 ppm
C)
—CH2—
acyl C-4 to C-(ω3) saturated chains
—CH3
alkyl chain
acyl chain
—CH2—
-CO-CH2
CH2-CH=CH-
CO-CH2-CH2
2.5 2.4 2.3 2.2 2.1 2.0 1.9 1.8 1.7 1.6 1.5 1.4 1.3 1.2 1.1 1.0 0.9 0.8 0.7 0.6 ppm

Figure 2.

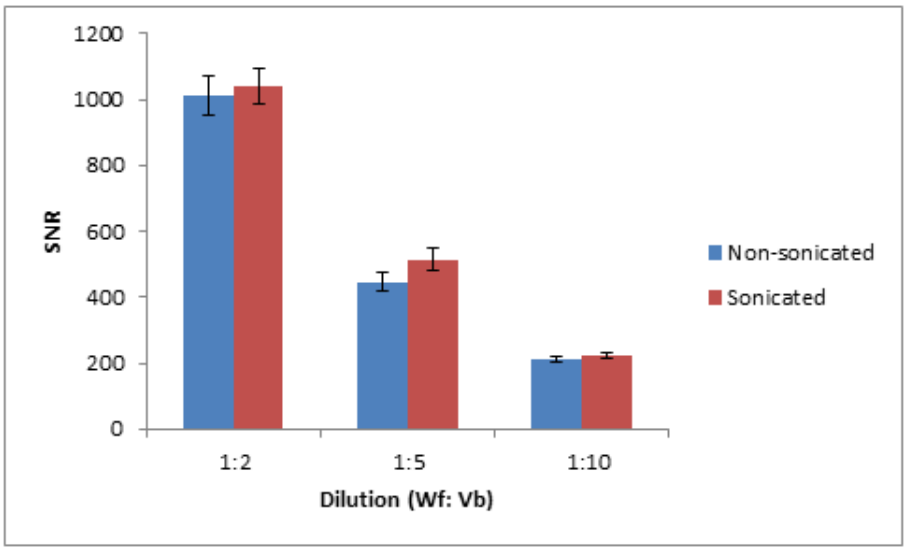
1200
1000
800
600
400
200
0
SNR
1:2
1:5
1:10
Dilution (Wf: Vb)
Non-sonicated
Sonicated

Figure 3.

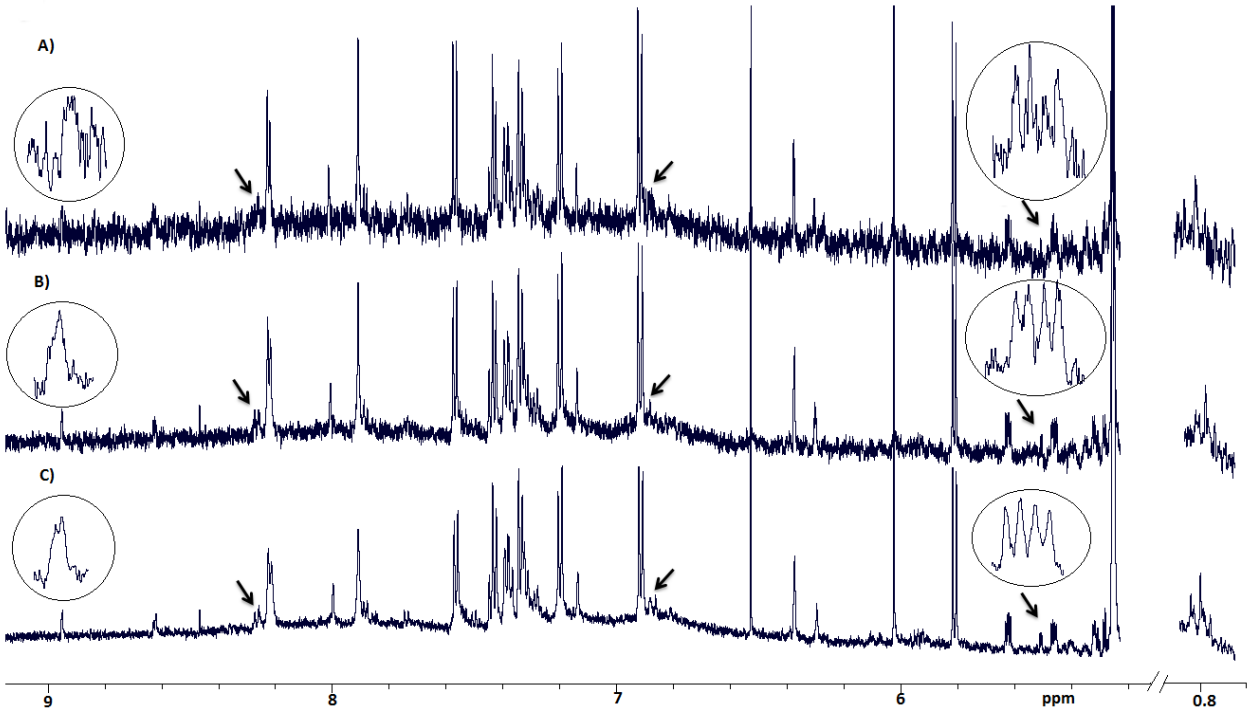

Figure 4.

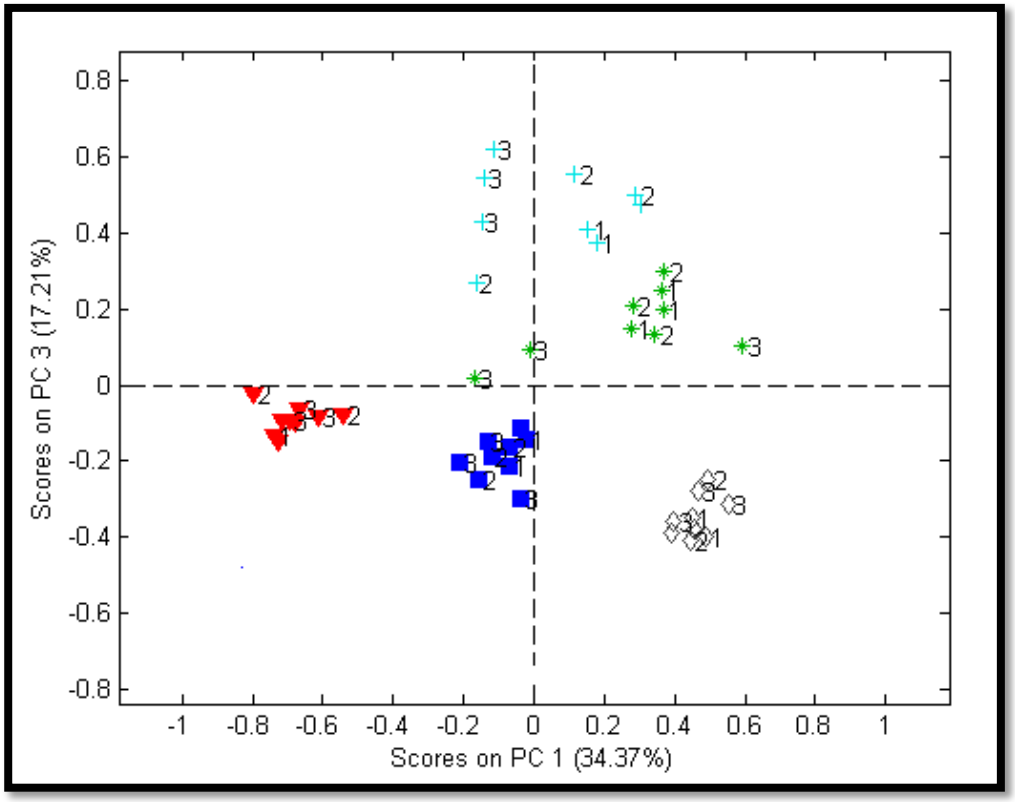
Scores on PC 3 (17.21%)
Scores on PC 1 (34.37%)
0.8
0.6
0.4
0.2
0
-0.2
-0.4
-0.6
-0.8
-1
-0.8
-0.6
-0.4
-0.2
0
0.2
0.4
0.6
0.8
1

Figure 5.

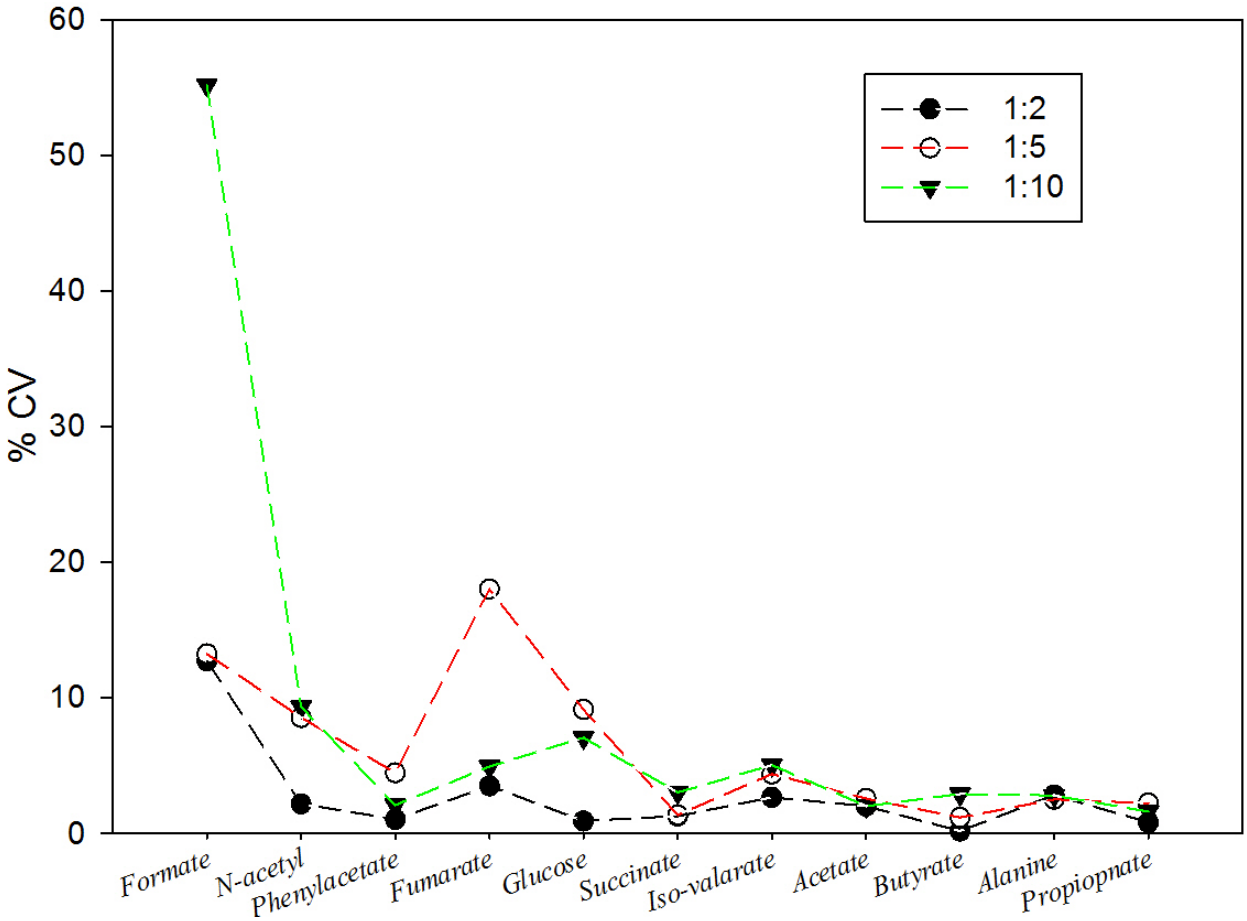
60
50
40
30
20
10
0
% CV
1:2
1:5
1:10
Formate
N-acetyl
Phenylacetate
Fumarate
Glucose
Succinate
Iso-valarate
Acetate
Butyrate
Alanine
Propiopnate

Figure 6.

Table 1. [1]H NMR Chemical Shifts and Assignments of Metabolites Detected in Human Fecal Extracts

No.	Compound	Proton Chemical shift in ppm and multiplicity
1	2-methyl butyrate [a]	0.86 (t), 1.05 (d), 1.38 (m) , 1.5 (m), 2.21 (m)
2	caprylate	0.85 (t), 1.27(m), 1.53 (m), 2.16(t)
3	valerate	0.88 (t), 1.31(m), 1.54 (m), 2.18 (m)
4	n-butyrate	0.91 (t), 1.56(t), 2.16 (t)
5	leucine	0.96 (d), 0.97(d), 1.7 (m)
6	iso-leucine	0.94 (t), 1.02 (d), 1.27 (m)
7	valine	0.99 (d), 1.05(d), 2.3 (m)
8	propionate	1.06 (t), 2.19 (m)
9	methyl succinate	1.08 (d), 2.11 (dd), 2.51 (d), 2.61 (m)
10	iso butyrate	1.12 (d), 2.40 (m)
11	ethanol	1.18 (t), 3.6 (q)
12	3-methyl 2oxovalerate [a]	0.89 (t), 1.10 (d), 1.46 (m), 1.70 (m), 2.93 (m)
13	threonine	1.33 (d), 3.57 (d), 4.24 (m)
14	lactate	1.31 (d), 4.1 (q)
15	acetoin	1.37 (d), 2.20 (s), 4.42(q)
16	iso-caproate	0.88 (d), 1.45 (m), 1.49 (m), 2.19 (t)
17	alanine	1.48 (d), 3.78 (q)
18	cadavarine	1.46 (m), 1.73 (m), 3.02 (m)
19	lysine	1.40 (m), 1.70 (m), 3.03 (t), 3.72 (t)
20	unknown singlet	1.81 (s)
21	thymine	1.87 (s), 7.39(s)
22	arginine	1.68 (m), 1.91 (m), 3.23 (t), 3.76 (t)
23	acetate	1.92 (s)
24	n-acetylglutamate	2.02 (s), 2.05 (m),4.1 (m)
25	iso-valerate	2.06 (d), 1.98 (m), 0.91 (d)
26	glutamate	2.14 (m), 2.46 (m), 3.78 (m)
27	succinate	2.41 (s)
28	glutamine	2.12 (m), 2.44 (m), 3.75 (t)
29	5 aminopentonate [a]	3.02 (t), 2.21 (t), 1.69 (m), 1.65 (m)
30	methionine	2.15 (m), 2.63 (t), 3.85 (dd)
31	sarcosine [a]	2.72 (s), 3.6 (s)
32	aspartate	2.69 (dd), 2.82 (dd), 3.91 (dd)
33	trimethylamine	2.87 (s)
34	3-phenylpropionate [a]	2.87 (t), 2.47 (t), 7.26 (t), 7.32 (d), 7.36 (t)
35	malonate	3.11 (s)
36	choline	3.27 (s), 3.53 (m)
37	proline	1.98 (m), 2.06 (m), 2.34 (m), 3.32 (dt), 3.34 (m)
38	ornithine	1.72 (m), 1.82 (m), 1.93 (m), 3.03 (t), 3.77 (t)
39	glucose	3.40 (t), 3.53 (dd), 3.71 (t) 3.73 (dd), 3.83(m), 5.24(d)
40	glycerol	3.64 (m), 3.78 (m)
41	glycine	3.57 (s)
42	ribose	3.52 (m), 3.60(m), 3.91 (m), 4.10 (m), 4.20 (dd), 4.92 (d), 5.25 (d),

43	dihydroxyacetone	4.41(s)
44	xylose	5.19 (d), 4.57 (d), 3.92(dd), 3.89(dd), 5.18(d), 3.31(d)
45	galactose	3.54(dd), 3.65(dd), 3.72(m), 3.82(m), 4.06(t), 5.26(d)
46	uracil	5.78 (d), 7. 49 (d)
47	fumarate	6.52 (s)
48	tyrosine	6.91 (d), 7.20 (d)
49	histidine	3.16 (dd), 3.23 (dd), 3.98 (dd), 6..99 (d), 7.83 (s)
50	phenylalanine	3.54 (s), 7.31 (m), 7.37 (m)
51	xanthine	7.94 (s)
52	hypoxanthine	8.12 (s), 8.21 (s)
53	formate	8.46 (s)
54	nicotinate	7.56 (dd), 8.28 (m), 8.66 (dd), 8.94 (d)
55	p-cresol	2.25 (s), 6.87 (m), 7.18(d),
56	putresine	1.77 (m), 3.06 (m)
57	urocanic acid [a]	6.38 (d), 7.27 (d), 7.87 (s)
58	dimethylamine	2.74 (s)
59	3 hydoxyphenlyacetate [a]	3.56 (s), 6.93 (m), 7.21 (m)
60	tryptophan	7.19 (m), 7.31 (m), 7.53 (d), 7.76 (d)
61	bile acid	0.78 (m)
62	UDP glucose [a]	5.62 (dd), 6.0 (m),
63	glutarate	1.8 (t), 2.19 (t)
64	malic acid	2.33 (dd), 2.65 (dd), 4.28(dd)
65	orotic acid	6.19 (s)
66	n-acetyl group's	2.04 (s).7,88 (s)
67	ethanolamine	3.12 (t), 3.80 (t)
68	allantion [a]	5.38 (s)
69	glycerophosphocholine	3.19 (s)
70	arabinose	5.24(d), 4.52 (d), 3.95(m), 3.89(m), 3.52(dd)

[a] Assignment with best matched signals

Table 2. Comparison of Yields of Standard Compounds with Different Wf: Vb during Extraction

Standards	Wf:Vb	Wf:Vb	Wf:Vb
	1:2	1:5	1:10
Sucincate	3.06±0.02	3.11±0.04	3.06±0.01
Dimthylsulphone	3.51±0.05	3.48±0.02	3.50±0.05

Means (±) SD determined from the integrals of the compounds relative to that of maleic acid

References

(1) Averna, T. A.; Kline, E. E.; Smith, A. Y.; Sillerud, L. O. *J. Urol.* **2005**, *173*, 433-438.

(2) Beckonert, O.; Keun, H. C.; Ebbels, T. M.; Bundy, J.; Holmes, E.; Lindon, J. C.; Nicholson, J. K. *Nat. Protoc* **2007**, *2*, 2692-2703.

(3) Bertram, H. C.; Eggers, N.; Eller, N. *Anal. Chem* **2009**, *81*, 9188-9193.

(4) Bertram, H. C.; Malmendal, A.; Petersen, B. O.; Madsen, J. C.; Pedersen, H.; Nielsen, N. C.; Hoppe, C.; Mølgaard, C.; Michaelsen, K. F.; Duus, J. Ø. *Anal. Chem* **2007**, *79*, 7110-7115.

(5) Bäckhed, F.; Ding, H.; Wang, T.; Hooper, L. V.; Gou, Y. K.; Nagy, A.; Semenkovich, C. F.; Gordon, J. I. *PNAS* **2004**, *101*, 15718-15723.

(6) Gao, X.; Pujos-Guillot, E.; Sebedio, J. L. *Anal. Chem* **2010**, *82*, 6447-6456.

(7) Hooper, L. V.; Midwedt, T.; Gordon, J. I. In *Ann. Rev. Nutr.*, 2002, pp 283-307.

(8) Nicholson, J. K.; Holmes, E.; Kinross, J.; Burcelin, R.; Gibson, G.; Jia, W.; Pettersson, S. *Science* **2012**, *336*, 1262-1267.

(9) Turnbaugh, P. J.; Ley, R. E.; Mahowald, M. A.; Magrini, V.; Mardis, E. R.; Gordon, J. I. *Nature* **2006**, *444*, 1027-1031.

(10) Rath, C. M.; Alexandrov, T.; Higginbottom, S. K.; Song, J.; Milla, M. E.; Fischbach, M. A.; Sonnenburg, J. L.; Dorrestein, P. C. *Anal. Chem* **2012**, *84*, 9259-9267.

(11) Gregory, K. E.; Bird, S. S.; Gross, V. S.; Marur, V. R.; Lazarev, A. V.; Walker, W. A.; Kristal, B. S. *Anal. Chem* **2013**, *85*, 1114-1123.

(12) Rezzi, S.; Ramadan, Z.; Fay, L. B.; Kochhar, S. *J. Proteome Res.* **2007**, *6*, 513-525.

(13) Lamichhane, S.; Yde, C. C.; Forssten, S.; Ouwehand, A. C.; Saarinen, M.; Jensen, H. M.; Gibson, G. R.; Rastall, R.; Fava, F.; Bertram, H. C. *J Agri Food Chem* **2014**, *62*, 9944-9951.

(14) Le Gall, G.; Noor, S. O.; Ridgway, K.; Scovell, L.; Jamieson, C.; Johnson, I. T.; Colquhoun, I. J.; Kemsley, E. K.; Narbad, A. *J. Proteome Res.* **2011**, *10*, 4208-4218.

(15) Marchesi, J. R.; Holmes, E.; Khan, F.; Kochhar, S.; Scanlan, P.; Shanahan, F.; Wilson, I. D.; Wang, Y. *J. Proteome Res.* **2007**, *6*, 546-551.

(16) Monleón, D.; Morales, J. M.; Barrasa, A.; López, J. A.; Vázquez, C.; Celda, B. *NMR Biomed.* **2009**, *22*, 342-348.

(17) Martin, F. P. J.; Sprenger, N.; Montoliu, I.; Rezzi, S.; Kochhar, S.; Nicholson, J. K. *J. Proteome Res.* **2010**, *9*, 5284-5295.

(18) Jacobs, D. M.; Deltimple, N.; van Velzen, E.; van Dorsten, F. A.; Bingham, M.; Vaughan, E. E.; van Duynhoven, J. *NMR Biomed.* **2008**, *21*, 615-626.

(19) Saric, J.; Wang, Y.; Li, J.; Coen, M.; Utzinger, J.; Marchesi, J. R.; Keiser, J.; Veselkov, K.; Lindon, J. C.; Nicholson, J. K.; Holmes, E. *J. Proteome Res.* **2008**, *7*, 352-360.

(20) Pettersson, J.; Karlsson, P. C.; Choi, Y. H.; Verpoorte, R.; Rafter, J. J.; Bohlin, L. *Biol. Pharm. Bullet.* **2008**, *31*, 1192-1198.

(21) Ndagijimana, M.; Laghi, L.; Vitali, B.; Placucci, G.; Brigidi, P.; Guerzoni, M. E. *Int. J. Food Microbiol.* **2009**, *134*, 147-153.

(22) Romick-Rosendale, L. E.; Goodpaster, A. M.; Hanwright, P. J.; Patel, N. B.; Wheeler, E. T.; Chona, D. L.; Kennedy, M. A. *Magn. Reson. Chem.* **2009**, *47*, S36-S46.

(23) Zhao, Y.; Wu, J.; Li, J. V.; Zhou, N. Y.; Tang, H.; Wang, Y. *J. Proteome Res.* **2013**, *12*, 2987-2999.

(24) Wu, J.; An, Y.; Yao, J.; Wang, Y.; Tang, H. *Analyst* **2010**, *135*, 1023-1030.

(25) Wishart, D. S.; Knox, C.; Guo, A. C.; Eisner, R.; Young, N.; Gautam, B.; Hau, D. D.; Psychogios, N.; Dong, E.; Bouatra, S.; Mandal, R.; Sinelnikov, I.; Xia, J.; Jia, L.; Cruz, J. A.; Lim, E.; Sobsey, C. A.; Shrivastava, S.; Huang, P.; Liu, P.; Fang, L.; Peng, J.; Fradette, R.; Cheng, D.; Tzur, D.; Clements, M.; Lewis, A.; de souza, A.; Zuniga, A.; Dawe, M.; Xiong, Y.; Clive, D.;

Greiner, R.; Nazyrova, A.; Shaykhutdinov, R.; Li, L.; Vogel, H. J.; Forsythei, I. *Nucleic Acids Res.* **2009**, *37*, D603-D610.

(26) Bojstrup, M.; Petersen, B. O.; Beeren, S. R.; Hindsgaul, O.; Meier, S. *Anal. Chem.* **2013**, *85*, 8802-8808.

(27) Savorani, F.; Tomasi, G.; Engelsen, S. B. *J. Magn. Reson.* **2010**, *202*, 190-202.

(28) Harwood L.J ; Weselake J. R, ^{1}H NMR spectroscopy of fatty acids and their derivatives [Online] **2005,** http://lipidlibrary.aocs.org/nmr/1NMRintr/index.htm (accessed Feb 26, 2015).

(29) Willker, W.; Leibfritz, D. *Magn. Reson. Chem* **1998**, *36*, S79-S84.

(30) Pulkkinen, M. O.; Nyman, S.; Hamalainen, M. M.; Mattinen, J. *Gynecolo Obstet Invest* **1998**, *46*, 220-224.

(31) Vu, T. N.; Laukens, K. *Metabolites* **2013**, *3*, 259-276.

(32) Alonso, A.; Rodriguez, M. A.; Vinaixa, M.; Tortosa, R.; Correig, X.; Julia, A.; Marsal, S. *Anal. Chem* **2014**, *86*, 1160-1169.

(33) Lewis, S. J.; Heaton, K. W. *Scand. J Gastro.* **1997**, *32*, 920-924